Environmental Biology and Chemistry Branch:
Summary Statement

by Dr. J. A. Moore

The Environmental Biology and Chemistry Branch focuses on three objectives: to maintain collaborative support laboratories in the disciplines of Chemistry, Inhalation Toxicology, Comparative Pathology, and Veterinary Medicine; to program and coordinate experimental animal procurement, housing, and utilization within the Institute; to plan and conduct research appropriate to the capabilities of Branch laboratories.

Administratively, the Environmental Biology and Chemistry Branch is organized into four sections: Animal Husbandry, Chemistry, Comparative Biology, and Inhalation Toxicology. Operationally, specific work units (size and composition of each being determined by task requirements) often are drawn from several sections of the Branch.

Animal Husbandry

A major activity during the year was the completion of design and specifications for research animal quarters and support laboratories within the NIEHS permanent site. A development plan for staffing the facility, including a review of current practices is being developed; this plan will allow us to initiate long-lead activities before we occupy completed facilities.

Allocation of animal space continues to be guided by the NIEHS Animal Experimentation Committee. Lack of adequate animal space still continues to be a limiting factor in some phases of the Institute's research program. There are plans for construction and occupancy of temporary animal holding quarters in FY 1978. This facility will allow us to relocate breeding colonies from within Building 15 and subsequently allocate this space for general experimentation.

The Animal Husbandry Section continued to develop and refine competence in a variety of technical animal procedures. The Section routinely provides capabilities in the areas of test diet preparation and feeding, animal breeding (including timemating and maintenance of inbred stocks), as well as technical services such as administration of test chemicals, forced breeding, and necropsy assistance.

The quality of animals received at NIEHS was systematically reviewed during the year. Plans for utilizing animals of a defined flora rather than current commercial sources is being actively considered. A major concern remains that of adequate transportation from the site of production to the laboratories in Research Triangle Park, N. C. Major strides in this problem area were realized through initiating direct transport of random bred mice, rats and guinea pigs by the producers' climate controlled truck to the Research Triangle Park. Shipment of inbred mice still remains a problem.

A review of experimental animal diets used at NIEHS led to a decision to utilize an open formula rodent diet. Such a diet insures constancy of food ingredients and sources. Rigid quality control of trace contaminant levels of metals and pesticides continued to reveal NIEHS diets to be of uniformly high quality. Instituting controlled shipment from the milling site to NIEHS enhanced diet quality through elimination of major transport variable associated with temperature and sanitation.

Chemistry

Environmental Chemistry is the branch of chemistry which deals with the behavior of environmental agents in the environment and their effects on the physical environment and the biosphere. It has a particular focus in this Institute on delineating the chemistry of environmental agents which show (or have the potential to) biological or otherwise deleterious effects on mammalian systems as animal models for man with particular emphasis on manmade (xenobiotic) persistent molecules. The Chemistry Section continues to pay

particular attention to the halogenated organics and the lipophilic xenobiotics in general.

Support Activities

Environmental chemistry interfaces environmental analysis, the actual detection and measurement of environmental agents in the environment, with environmental toxicology, the detection and measurement of various deleterious biological activities of environmental agents. Therefore, chemists are frequently consulted within NIEHS in order to advise biological scientists in appropriate areas of their research. The support activities of the Chemistry Section as normal, fluctuate depending upon the resources required to meet a particular need. Chemistry support requests are progressively increasing both in terms of the number and the degree of involvement, and the requests generally fall into one of two categories: short-term and to-the-point requests such as compound purity determinations and organic synthesis by published procedures, and a variety of long term requests which generally require new and improved methodology development. In the latter case it is difficult to determine the amount of time and effort which will be necessary to complete the task. A trial period may be necessary to demonstrate feasibility within the limitations of the group. Therefore, for obvious reasons these latter requests are carefully evaluated, and generally we seek to do this type of work on a collaborative research support basis.

As the Institute has begun to grow in anticipation of occupying the permanent facility, it has been necessary to provide a better definition of the chemistry support role. Chemistry support will generally involve the use of established known methodologies in all cases as opposed to the development of new ones. We will consider requests where new methods are required when it appears to be particularly relevant to ongoing programs within the group and/or otherwise generally in keeping with the expertise of the group. Special considerations will be made where sufficient research priority exists to do things which cannot be done at all on the outside or only very slowly, or where it is clear that we can probably do it better. A number of specific routine capabilities have been identified and are available for chemistry support purposes. These services can be requested through initiation of the routine chemistry service request form. Acceptable requests are generally handled on a first-come, first-served basis. In addition, the Office of the Head will provide support in securing procurement and research contracts of a chemical nature. Chemistry support requests are received and reviewed by the Chemistry Section Office and then passed on to one of three work groups best suited to handle the request. These work groups also form the basis for research activities within the Section. The three work groups are: Analytical Biochemistry, Bioorganic Chemistry and Synthesis, and Specialty Instrumentation. A number of other capabilities exist within the Chemistry Section which are sufficiently time consuming to require a priority collaborative research protocol to engage.

The largest number of analytical runs (over 2000) was made by the Specialty Instrumentation Group using gas chromatography-mass spectrometry (GC/MS). Of these, about 30% were routine service requests, about 44% in collaborative support of various biological studies, and about 26% for instrumental and method development research. About 40% of the total runs supported various projects within the Chemistry Section. Nuclear magnetic resonance spectroscopy (NMR) was used on a limited number of samples for further characterization. Demands for high pressure liquid chromatography (HPLC) analysis also increased considerably and involved over 400 sample runs. The samples run included a variety of chemical standards, actual metabolites and other trace residues which occur in a milieu providing interference. One of the more involved collaborative research requests involving the Specialty Instrumentation Group resulted in the identification of the metabolites of 4-chlorobiphenyl and 4,4′-dichlorobiphenyl in the rat.

NMR was used to analyze a variety of chemical and biological metabolites and in many both ^{1}H and ^{13}C nuclei were observed using ^{1}H decoupling and Fourier Transform (FT) techniques. Most of these samples were generated from other projects within the Section. For example, by using ^{13}C and ^{1}H NMR, substitution patterns of the major brominated biphenyl components of the flame retardant mixture Firemaster FF-1 were identified as 2,4,5,2′,4′,5′-hexabromobiphenyl and 2,3,4,5,-2′,4′,5′-heptabromobiphenyl. Patterns of other minor isomers have also been identified in this manner. ^{13}C NMR spectra of a number of potential oxidative metabolites of benzo[a]pyrene are being obtained to aid in identification of these compounds in biological and environmental studies. The more difficult synthetic and/or preparative (using HPLC) requests included the two major brominated biphenyl components of Firemaster FF-1 and the phananthrene derivative of diethylstilbestrol and 2,4-oxazolidinedione.

A number of other requests were made for analytical services which required specific methods. These ranged from specific analysis for various enzymes, lipids, urinary calculi, silicate contents, specific isomer content, metabolites, and

purity and radiopurity analysis of unlabeled and labeled research chemicals to syntheses, biosyntheses, and determination of reaction rates and reactivity profiles. More involved requests included determinations of rates of hydrolysis and reactivity toward nucleophiles for various acylating agents under teratogenicity study, purity analysis and stability studies of dosing solutions of symmetrical dimethylhydrazine and determinations of ^{35}S Captan in embryonic tissue.

Progress in development of chemical techniques and analytical methods generally useful to the Institute and their unique application to specific classes of environmental chemicals under study are continually being reviewed and evaluated. As a result of the many diverse requests, research methodology is continually being developed in analytical biochemistry and metabolism organic chemistry and synthesis, and special instrumental analytical chemistry. Methods for the preparation, determination, and characterization of various compounds and compound classes will facilitate the successful elaboration of their chemistry and biochemistry and permit more definitive biological and toxicological studies.

Research Activities

The nature of the research in the Chemistry Section is to some degree, predetermined by its support commitment and size and space limitations. For example, the Chemistry Section attempts to provide broad areas of science competence rather than in-depth and narrow science expertise. However, to do this requires the availability of diverse instrumentation which consumes space and requires routine maintenance. There are five major areas of research involvement in the Chemistry Section which are using selected classes of chemical compounds as models and/or special chemical, biochemical and instrumental techniques to develop general methodology of value to environmental health scientists:

(1) The first is seeking to develop immunoassay methods which will offer extreme specificity and sensitivity to the detection of hazardous environmental agents and their residues themselves as well as their biological insults, such as mutated proteins, at biologically meaningful levels.

A variety of hydrophobic aromatic halogenated haptens have been used to produce defined antigens. Antibodies to appropriately prepare antigens can generally be raised with no great difficulty. Polybrominated haptens have been shown to yield relatively ineffective antigens. Adsorbed haptens must be removed from the antigens for successful immunogenicity of the latter. Procedures for characterization and insuring the reproducibility of the antigens have been worked out. Utilization of the antisera and development of immunoassays are beginning. Sensitivity of immunoassays may be less of a problem than specificity for these types of compounds. It has also been necessary to investigate means of rapid processing of tissues and other environmental samples in regard to the unique problems associated with hydrophobic haptens.

(2) A second major effort has involved the chemical analysis and the toxicopathologic and pharmacologic effects of polychlorinated biphenyls (PCBs). In particular, the symmetrical hexachlorobiphenyls (HCBs) as representative model PCBs with high and constant chlorine content permitting unequivocal study of a given substitution pattern have been studied in chicks and mice. Previous studies have shown separate and distinct differences in isomer toxicity. These studies have been extended to include the one remaining untested symmetrical isomer and several unsymmetrical isomers including potential metabolite possibilities and an isomer containing the least toxic and most toxic substitution pattern in one molecule, and an isomer containing the most toxic pattern in both rings and one additional *ortho* chlorine affecting planarity of the molecule. Differences in isomer toxicity observed can be related to chemical structure *via* the affects of varying chlorine substitution on compound lipophilicity and metabolism. For example, preliminary studies have established that one of the mechanisms whereby a polychlorinated biphenyl becomes bound to biological systems is through the formation of a charge transfer complex. This finding implicates aromatic amino acids and the purine bases as possible binding sites for the PCBs. Significant differences in binding propensity for two of the symmetrical hexachlorobiphenyl isomers have been demonstrated to date.

A computer-assisted method for the identification of PCB isomers in a mixture requiring only minimal resolution of the mixture has been developed. Methods commonly used for the clean-up of environmental samples and tissue extracts have been evaluated relative to the quantitative determination of PCBs and chlorinated dibenzodioxins. A simple procedure for such clean-up has been proposed and supported by careful recovery studies.

The major associated impurities of PCBs, namely, the chlorinated dibenzofurans and chlorinated naphthalenes, are also being investigated. Selected isomers in the chlorinated naphthalene series are being synthesized. The Diels-Alder cycloaddition reaction has been utilized in two different ways to generate the naphthalene nucleus with substituents appropriate to further transformation to selected isomers. ^{14}C-Radiolabeled 2,3,7,8-tetra-

chlorodibenzofuran is also being synthesized for studies on species' differences in regard to variation in adsorption, metabolism, distribution and excretion. The first attempt at the radiosynthesis failed and appeared to be related to the high specific activity and accompanying radiochemistry of the phenolic precursor compound used. The synthetic reaction scheme incorporating the label in the other precursor compound, ^{14}C-labeled bromobenzene, will be repeated.

(3) A third major effort is pioneering the application of chemical ionization mass spectrometry to environmental problems. Modification of our existing high pressure chemical ionization mass spectrometer has been completed which will permit the formation and observation of either positive or negative ion beams. In the negative ion mode, a considerable enhancement in sensitivity to halogenated hydrocarbons has been realized; thereby facilitating their trace level detection in biological environmental samples. Specificity has also been realized in this mode of operation when applied to the various available chlorinated dibenzo-*p*-dioxins. When oxygen is used as a reagent gas, two types of product ion formation occur: the displacement of chlorine by oxygen from the molecular anion, and the formation of a quinoxide anion. The chlorine displacement reaction predominates for all isomers containing a *peri* chlorine. The unique sensitivity and specificity of the technique has been successfully applied to the quantitative analysis of chlorinated dibenzo-*p*-dioxins in dairy cow tissue extracts. These dioxins were also found in the pentachlorophenol treated lumber from the barns used to house these animals.

In the positive ion mode of operation, biacetyl was shown to be a useful reagent gas giving an acetylated product ion with a variety of functionally different organic molecules. This reagent was shown to preferentially acetylate the less sterically crowded isomer in a number of 4-*tert*-butylcyclohexyl esters as well as norbornediol esters.

(4) A fourth major involvement continues to be the development of special instrumental analytical methodology for application to biological problems at the mechanistic (molecular) level. Currently being emphasized is the modification/update of our Fourier Transform Nuclear Magnetic Resonance (FTNMR) spectrometer. Major modifications to the XL-100 NMR system have resulted in an increase in sensitivity and extension of the capabilities of the instrument. The addition of a new magnet power supply and probe allows the use of 10-mm sample tubes that in turn results in a greater than 2-fold improvement in sensitivity for ^{1}H and ^{13}C over the previous configuration. Installation of a single side band crystal filter improved the sensitivity by an additional factor of 2.5. The addition of a micro insert for ^{13}C NMR has allowed routine ^{13}C-NMR spectra to be obtained on 1-mg samples and even lower for weekend runs. Acquisition of the Gyro Code observe accessory extends the instrument's capability to include many of the heavy metal nuclei of present day interest. Essentially, NMR spectra may be obtained for any nucleus whose frequency of absorption is within the range of 17–40 MHz. Future updates of the instrument should include accessories to extend the above range of nuclei from 7 to 40 MHz and an 18-mm ^{13}C probe which would allow routine ^{13}C NMR spectra to be obtained on dilute samples and biological samples of limited solubility.

^{13}C-NMR has been used to characterize several chlorinated polycyclodiene pesticides and some of their phototransformation and potential metabolic products. It was shown to be superior to proton NMR for identification of these compounds and elucidation of their peculiar electronic interactions which may underlie their biological activity. In addition, NMR is being used to investigate the regiospecificity and stereospecificity of the chemical conjugation of specific ^{13}C-labeled benzo[a]pyrene oxides to glutathione, enzymatically and nonenzymatically.

(5) A fifth area of involvement seeks to determine the immunologic profile of various animals under study at the Institute as a part of the dose related spectrum of toxicologic effects. Ingestion of polybrominated biphenyls appears to exert toxicologic effects in man and animals. Results of our studies have indicated that *in utero* exposure to PBB in cattle induces at this particular time following exposure a state of hyperimmunity, especially with regard to cell mediated immunity. This was evidenced by a significant increase in mitogenic stimulation.

Successful treatment for exposure to 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in man and animals is evidently lacking. Attempts to passively immunize mice with antibodies to 2,3,7-trichlorodibenzo-*p*-dioxin one day prior and one day following treatment with TCDD have failed to show any protective effect. This was evidenced by no statistically significant effect on body weight loss and mortality.

Other Activities

The Chemistry Section continues to provide unique analytical chemistry support to the study of environmental contamination problems of imminent public health concern. For example, the Institute has embarked on an epidemiological study to

evaluate the health effects on infants of specific components of infant foodstuffs, particularly chlorinated pesticide residues and polychlorinated biphenyls (PCBs) in breast milk. In this regard, the Chemistry Section has reviewed and evaluated current methodologies for measuring total organic chlorine and PCB residues in body fluids and tissues. Specific methods have been identified, improved, and recommended for use by contractors in providing analytical chemistry support to this large study.

In another study, a dairy herd was found to be contaminated with chlorodibenzo-*p*-dioxins. Liver samples contained between 1 and 150 ppb octachlorodioxins, 0.01 to 20 ppb heptachlorodioxins and 0.01 to 1 ppb hexachlorodioxin. Somewhat lower levels were found in the fat.

In a third study, exposure to polybrominated biphenyls (PBBs) occurred through contaminated feed accidently shipped to two Farm Bureau Feed Mills in Michigan in 1973. The flame retardant PBB mixture FF-1 has been subjected to chemical and toxicological study. No evidence could be found for the presence of brominated dibenzo-*p*-dioxins or brominated dibenzofurans. The material analyzed was found to be contaminated with a few hundred parts per million bromonaphthalenes. By using chromatographic techniques, the Firemaster mixture could be separated into polar and nonpolar fractions. No evidence could be found to support the presence of highly toxic impurities in the polar fraction. The nonpolar fraction was found to have toxic properties similar to those of the crude material.

The Section Office continues to evaluate the cost versus procedure benefit relationships for various custom synthesized chemicals (labeled and unlabeled) obtained on contracts. Necessary water purification equipment to guarantee a specified water quality for laboratory animal drinking water is currently being fabricated by a contractor.

Invitations to present lectures, teaching assignments at local universities, and nominations to standing committees are accepted without rancor and are usually followed by active participation. The Chemistry Section is striving hard to establish a rapport with the various pertinent departments of the local universities in order to further enhance its research productivity and provide professional growth.

The Chemistry Section remains cognizant of the Institute Safety Program. A special hazardous chemical containment facility for the Section is in the fabrication stages. The facility will permit a variety of chemical operations involving hazardous chemicals to be conducted with the minimum exposure possible to laboratory personnel. The Section Office continues to cooperate with the safety office in design of specific chemical disposal procedures as needed.

Comparative Biology

The research of the Comparative Biology Section continued to focus on general toxic effects associated with halogenated polycyclic compounds such as chlorinated dibenzodioxins, chlorinated dibenzofurans, polychlorinated biphenyls and polybrominated biphenyls.

Study of the toxicity of chlorinated dibenzofurans was extended to the primate species. The LD-50 of 2,3,7,8-tetrachlorodibenzofuran (TCDF) in the rhesus monkey was found to be 1000 μg/kg. The pattern of toxicity observed in the primates was identical to that previously produced by the chlorinated dibenzodioxins. The failure to produce toxicity in mice and rats with 2,3,7,8-TCDF still remains unexplained. Further studies in these species are delayed pending receipt of radiolabeled compounds being synthesized under contract. Difficulties in synthesis have occurred. Studies of the comparative toxicity of the dibenzofurans will proceed once appropriate labelled TCDF is available. Initial plans are to determine if different patterns of distribution, metabolism or absorption occur in these species.

To further investigate the mechanism of toxicity due to chlorinated dibenzodioxins, a series of experiments were performed that define the effects of TCDD on fat absorption, metabolism, and transport of nutrients from the intestine. In these studies, rats which had received a lethal dose of TCDD were found to have a reduced rate of fat absorption from the small intestine as early as 3 days post-TCDD exposure. It was further observed that the absorbed globules remained within the intestinal epithelial cells longer and by ultrastructural analysis the lipid globules are poorly transferred out of the cell. These studies are still in progress.

Previous experiments with TCDD indicated that exposure to very low doses markedly increased the suspectibility of mice to Salmonella challenge. These studies suggested that increased mortality due to Salmonella could be associated with macrophage function. Recent experiments that focused on macrophage function found no major alteration: the rate of phagocytosis was not impaired, the number of cells capable of phagocytosis and bactericidal capability of macrophage was not altered. There was, however, a decrease in the numbers of macrophages available as determined by the number of leucocytes induced to migrate into the peritoneal cavity following exposure to thioglyco-

late. The numerical reduction in macrophage numbers was of sufficient magnitude to account for the increased mortality due to Salmonella challenge.

Experiments on the effect of environmental chemicals on immunocompetence expanded through study of environmental chemicals such as lead, diethylstilbestrol, and methyl butyl ketone. Results of previous studies with TCDD were confirmed and extended. Animals exposed prenatally and postnatally to TCDD have depressed body weights and depressed cellular immune responses. This depression is not lifelong. Responses returned to normal by 7 months of age. The T-cell-mediated humoral immune responses of TCDD-exposed rats are not suppressed, indicating that all T-cell subsets are not altered. In contrast, studies with lead have shown that pre- and postnatal exposure causes suppression of immune competence of both the cell mediated and humoral immunity. The studies with methyl butyl ketone are in progress. This work does clearly extend to a variety of chemicals the finding that environmental contaminants may exert subtle effects on man and animals, and that among these effects may be a modulation of the immune response either through suppression or enhancement.

An extensive toxicologic evaluation of Firemaster FF-1, the polybrominated biphenyl (PBB) which resulted in widespread contamination in Michigan, is in progress. These studies represent a collaborative effort that involves several laboratories within the Institute. A subsequent 6-month experiment will allow for more precise characterization of toxic effects; assess the toxicity associated with chronic exposure and provide the data base from which to determine doses to be used in carcinogen bioassay performed by the National Cancer Institute.

Inhalation Toxicology

The program of the Inhalation Toxicology Section is composed of three parts. (1) Studies are conducted of compounds to which toxicologically significant exposure would be expected to be primarily by inhalation. Research is focused on expressions of toxicity at the levels of tissues, organs, and organ systems. Emphasis is placed on interactions among the cardiovascular system, the lungs, kidneys, and liver. (2) A principal effort of the Section is directed toward the advancement of inhalation technology. (3) Support is provided to intramural scientists outside the Section in the form of consultation, and collaboration, where appropriate, in matters of inhalation toxicology. The Section also allocates up to 50% of its small animal inhalation exposure chamber capacity to studies originating outside the Section as a service.

All work in inhalation research and technology during the past year has been devoted to inhalation of gases. We expect this to be extended to particulates as necessary resources become available. Studies are continuing of low molecular weight halogenated alkanes and ethers such as solvents, refrigerants, and inhalation anesthetics. The program is in the process of being expanded to include simple amines also used as solvents, as well as certain amines associated with the processing of coal and oil that might be expected to pose hazards through inhalation or ingestion.

Chronic intermittent exposure to relatively low concentrations of chlorodifluoromethane have been reported to be responsible for the appearance of superventricular arrythmias in certain occupational settings. Efforts are underway to establish an animal model of this phenomenon for the purpose of investigating the mechanism of this unique action among the halogenated alkanes. Although the conditions necessary to reproduce reliably this phenomenon in an experimental animal have not yet been completely defined, evidence for liver damage by this compound has been regularly observed.

Indocyanine green (ICG) is being reevaluated as a diagnostic agent for evaluating toxic injury to the liver. The observation that plasma ICG decay rates are relatively insensitive indicators of hepatic dysfunction in the rabbit, is consistent with the observations of others. We do have evidence, however, of an enterohepatic circulation of ICG in the rabbit which is in disagreement with the published findings of other workers. We are tentatively planning to evaluate fluorescein after the model of ICG.

Exposure of rats to 2% vinyl bromide by inhalation results in the accumulation of a pharmacologically significant body burden of inorganic bromide. The CNS depressant effect of the inorganic bromide liberated during vinyl bromide exposure is mimicked by the administration of potassium bromide in the drinking water. This observation supports the conclusion that the effect is attributable to the bromide rather than other products of biotransformation. Most of the weight loss observed during the vinyl bromide exposures was attributed to reduced feed intake rather than specific toxic effects of the compound.

Measurements of pulmonary flow resistance, compliance, and carbon monoxide capacity have been performed on several species of small laboratory animals. The magnitude of the measured variables correlated well with body weight supporting the utility of these measurements in evaluating pulmonary function in inhalation toxicology research.

The hydroxyapatite of bone is an avid sink for inorganic fluoride. The procedure has been published by others in which the femurs of mice ex-

posed to fluoroorganic compounds are assayed for fluoride content as an index of defluorination. This method is being adapted for use in our laboratory.

A laboratory has just been completed for the study of the effects of toxic gases on the electromechanical performance of muscle, particularly myocardium. Studies are conducted on anesthetized open chested small laboratory animals, and on isolated perfused whole heart as well as tissues such as papillary muscles.

Another laboratory is being established for the purpose of studying lipoprotein surfactant, lung cells, macrophages, and a variety of enzymes associated with pulmonary lavage. This facility will be devoted to work in conjunction with the pulmonary function testing group.

A highly automated monitoring and control system for a multichamber small animal inhalation exposure facility is being built. Data representing the exposure conditions in the respective chambers are automatically acquired.

The data are used for two purposes: logging and display for use by the principal investigators and control of the chambers through a feed-back system. Calibration routines are employed in which the operator interacts with the controller. Chamber operation is then placed under program control which includes automatic start-up, pre-programmed exposure profile generation, and shut-down of the chambers at the prescribed time. Error detection systems alert the operator to problems outside the controller's capability.